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BACTERIAL LEAFSPOT OF CLOVERS[1]

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INTRODUCTION

A leafspot disease of red clover, *Trifolium pratense*, quite unlike any of the several well-known diseases of this crop, was first noted in clover fields and on wayside plants in the vicinity of Madison, Wis., in 1916.[2] The next year the same disease was observed in the vicinity of Raleigh, N. C., not only on red clover but also on white clover, *Trifolium repens*, and on alsike, *Trifolium hybridum*, as well (Pl. 1, 2). This disease has been found in the District of Columbia and in neighboring Virginia and Maryland fields on the common red and white clovers. At Arlington, Va., it also occurs on *Trifolium repens*, var. *latum*, *Trifolium medium*, *Trifolium hybridum* and *Trifolium pannonicum* (Pl. 3). Preliminary microscopic examinations made of the Wisconsin material in 1916 showed that the disease was probably of bacterial origin. This conclusion was supported by the fact that isolation then made yielded a preponderance of similar white, bacterial colonies. Transfers from these proved uniformly to be pathogenic on red clover. A survey of the literature on clover diseases, both American and foreign, was accordingly made in an attempt to identify this bacterial leafspot disease. It was apparent from the few publications on this subject that this disease can not with certainty be identified as any of those previously described. Because of the economic importance of clover as a forage and hay crop, therefore, and of the lack of knowledge of this disease, independent investigations were undertaken at the University of Wisconsin, at the North Carolina Agricultural Experiment Station, and in the Laboratory of Plant Pathology at Washington, D. C. These have for the most part been prosecuted independently, with numerous interruptions at each place,

[1] Accepted for publication July 3, 1923.

[2] The investigations of this clover disease have been involved with those of related diseases of other legumes at Wisconsin and elsewhere, and acknowledgement is due to several associates aside from the authors. The disease on red clover was first observed by Dr. A. G. Johnson and Dr. C. S. Reddy in 1916, and the first isolation of the causal organism was made by Doctor Reddy. These two men and Dr. F. R. Jones have supplied further data on the distribution on this host. The details of the Wisconsin studies during 1917–18 passed into the hands of Miss Florence Coerper and were carried on in connection with her related investigations of soybean bacteriosis. In 1919 Miss Maude Miller (Mrs. Williamson) succeeded her in handling these details. When it was learned that Dr. F. A. Wolf in North Carolina and Miss Lucia McCulloch in Washington, D. C., had each independently found and studied the same disease with supplementary host range, it was decided to correlate the results for joint publication. The final development of these plans has necessitated conferences, somewhat delayed publication, and in places has encumbered the text with details which might otherwise have been omitted. It is, however, believed that the scientific worth and convenience of such correlation as compared with independent reports justifies joint publication. The only personal regret to me in the outcome is that the position of my own name as senior author fails properly to indicate the indebtedness to my younger associates for most of the details of workmanship.—L. R. J.

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and the present paper, which embodies a correlated report of these results, has finally been compiled at the suggestion of the senior author. The aim has been to give an adequate account of the disease as observed independently at the three locations, together with the evidence as to its etiology and the characters of the pathogen.

HISTORY AND DISTRIBUTION

Observations throughout the period covered by these investigations show that the disease appears every year throughout the growing season but is not usually the cause of serious damage. It is not conspicuous in the field except in periods when moisture conditions are especially favorable for its development. Observations made in Wisconsin, both in the vicinity of Madison and elsewhere in the State, in various parts of Iowa and Indiana, and at a number of widely distant points in North Carolina, also in Virginia, Maryland, and the District of Columbia, indicate that this disease is of very general occurrence. It doubtless occurs widely at least in the United States and has heretofore escaped notice because of its confusion with other clover leafspot diseases.

Mention has been made in previously published accounts of several bacterial diseases of clover. The first of these was described in 1896 by Voglino (7).[3] This account deals with a leafspot disease which was rampant in several provinces of Italy on white clover, was common on *Trifolium resupinatum*, but rarely attacked red clover. It caused the formation of definite, usually numerous small black spots, most evident on the lower leaf surface. The floral parts were also involved and presented a similar diseased appearance. Voglino concluded that this disease not only materially reduced the yield of forage but also rendered it distasteful to grazing animals. The disease was ascribed by him to a bacterial parasite which he described as a new species, *Bacillus trifolii*. So far as can be determined, this disease has thus far not been reported outside of northern Italy.

Although this Italian disease and the bacteriosis under discussion have certain characters in common, they differ in several important features. In the first place, the cultural characters, then not regarded as of special significance but now considered essential to the exact description of species of bacteria, are almost wholly lacking in Voglino's description. Further, *Bacillus trifolii* is described as 0.5 to 5.0μ in length by 0.2 to 0.5μ in width and is thus more slender than the organism associated with the disease under discussion. In addition, it forms, as shown in Voglino's figures 10 and 11, drumstick-like spores, a character not possessed by the organism herein described. Then, too, even in the absence of type specimens with which to make direct comparison, his figure 1, which illustrated the character of the lesions on the foliage, leaves no doubt that Voglino's disease is distinct from the one dealt with in the present paper.

A disease reported from Italy in 1913 by Baccarini and his associates (1) under the name "incappucciamento" manifests itself in a very different manner from the leafspot disease described by Voglino. Since this malady was so severe in northern Italy as to cause a failure of the crop, a commission was assigned to investigate it. Doctor Bargagli, bacteriologist for this commission, believed it to be of bacterial origin. This clover disease is characterized by a general stunting of the above-ground portions of the plant, as indicated by the dwarfed, yellowish

[3] Reference is made by number (italic) to literature cited, p. 490.

leaves and inhibition of development of new vegetative and floral organs. Baccarini's brief account neither names the causal organism nor includes a description of it, but the disease manifestly has no relation to the American bacterial leafspot.

In his comprehensive account of the diseases of the clovers in Russia, Jaczewski (3) makes no mention of any form of clover bacteriosis in that country, but indicates his familiarity with those in Italy by reference to the two listed above. Aside from these investigations, bacteriosis of clover in Europe appears not to be recorded, and the only account of its occurrence in America is that included in Manns' (5) studies of the "streak" disease which is especially prevalent upon the sweet pea. This disease, which is attributed to *Bacillus lathyri* Manns and Taubenhaus, is recorded also as occurring on clover and certain other legumes. This bacillus is a yellow organism with very different morphological and cultural characters from the clover organism under present consideration. Furthermore, Manns' disease is characterized by longitudinal stem lesions, which may later involve the petioles and leaves, thus presenting symptoms very different from those of the bacterial leafspot described by the writers.

It is entirely probable that bacterial leafspot has been confused with the several fungous leafspots which, especially in their later stages, may be difficult to differentiate without the aid of a microscope. At any rate, it has not hitherto been clearly recognized and there are no unquestionable previous records of its occurrence.

APPEARANCE OF THE DISEASE

SEASONAL DEVELOPMENT

A week or two after the first clover leaves become green in spring bacterial leafspot begins to appear. In Wisconsin the infections have usually been noted in late May and early June upon the young leaflets. Even though clovers remain green throughout the winter in North Carolina, no evidence of bacterial leafspot has been found until the warm days of May. In the vicinity of Washington, D. C., infections have not been observed until late in May. If the weather continues moist, the disease progresses upward with the growth of the plant so that at blossoming time even the uppermost leaves may be conspicuously spotted (Pl. 1).

There is no evidence that the disease is systemic, and new infections may appear at any time when temperature and moisture conditions are favorable. In some seasons severe infection appears on the young growth following the cutting of the first crop and may be found commonly in the fields as late as September and October. In 1916 the disease was very abundant on both the first and second crops of clover at Madison, while in 1919 hot, dry weather checked the disease in early June, and scarcely any new infections appeared after that time. At Arlington, Va., the disease has appeared in three successive years on the second crops of clover.

SYMPTOMS

As the name implies, the most conspicuous lesions of this disease appear on the foliage, although stems, leaf petioles, stipules, and flower pedicels are also seriously involved. The presence of tiny translucent

dots on the lower leaf surface is the first indication of infection. These lesions enlarge and become more or less angular, since they are quite sharply delimited by the veins. Meanwhile, the centers of the spots become inky black; but the margins retain the water-soaked character even at maturity. The centers of old lesions on desiccation become dark brown and parchmentlike. The tissues outside the translucent border of the lesion become chlorotic, and badly spotted leaves are distinctly yellowish. The infections may be so abundant that large, irregular, dead areas are formed. There is a tendency for the central tissues of old

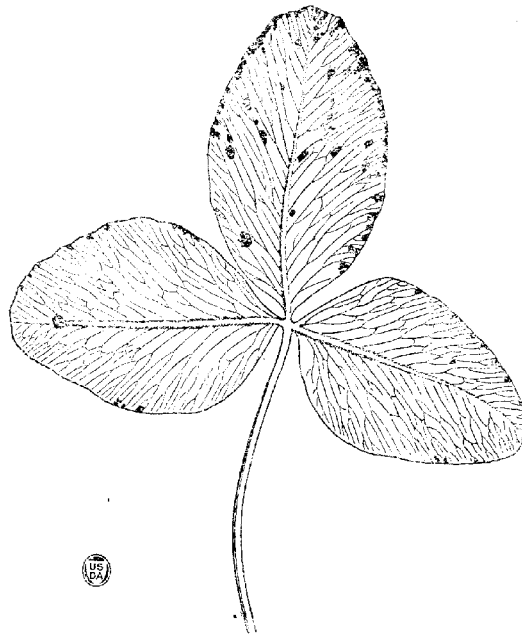

FIG. 1.—Leaf of red clover, *Trifolium pratense*, with bacterial leafspot, natural infection. This shows the characteristic form, size, and distribution of lesions, both marginal and interior, the latter generally intervenous. (Drawing from nature by Charles Drechsler.)

lesions to crack or to fall away. In consequence, if these lesions are marginal, the leaves present a frayed and torn appearance. Abundant spotting causes the entire leaflet to become dry or to shed prematurely.

Under very humid conditions a bacterial exudate may appear on the lower leaf surface. This exudate has the appearance of a thin film or of small, milky, glistening droplets. If diseased leaves are kept for 12 to 24 hours in a moist chamber, the bacterial slime will have exuded to form larger droplets from which the pathogen may be obtained in pure culture. On drying, this exudate becomes a thin, incrusting film.

Infection of petioles, stems, stipules, and flower pedicels are of less common occurrence than infection of the leaflets, and the lesions are less characteristic. Those of the petiole and stem appear as dark, elongated, slightly sunken spots. On stipules of *Trifolium pannonicum*, long, inky-black lines were produced (Pl. 3, D). The translucent margin is less pronounced than on leaf lesions.

CAUSAL ORGANISM

ISOLATION

Bacteria are abundant in the lesions, and at each of the three laboratories, Madison, Raleigh, and Washington, the identical organism has been isolated repeatedly. In some cases (Wisconsin, Washington) isolation has been preceded by surface rinsing of the tissues with alcohol or mercuric chlorid solution; in others (North Carolina) direct maceration was made of young lesions in sterile water. One of the early Wisconsin isolations from red clover, termed 1916–II, was first proved to be pathogenic, then used in the earlier detailed cultural studies, and more recently has been compared with the subsequent isolations from Madison red clovers, termed 1919–I and 1920–III. These three have been carried critically through comparative cultural studies at Madison and have throughout the experiments shown essentially identical characters.

FIG. 2.—Bacterium causing leafspot of red clover. Casares-Gil flagella stain.

At Raleigh several like strains were isolated from red, white, and alsike clovers and their pathogenicity proved by successful inoculation and reisolation. One strain from each of these host species was used for cultural studies at Roleigh in 1922. Other strains from isalations of previous years were also used in comparative studies with the Wisconsin strain 1920–III.

At Washington isolations were made from natural infections on the red, the common white, the large-leaved white (var. *latum*) and alsike clovers, also from *Trifolium medium* and *T. pannonicum*. All the strains were compared and found to be practically identical in morphological and cultural characters and in ability to reproduce the disease not only on the original host but also on other clover species. Three strains were selected for the further studies: Strain 3 from white clover, strain 4 from red clover, and strain M, a reisolation from red clover inoculated with strain 3.

MORPHOLOGY

The pathogen is a small rod with rounded ends, usually occurring singly but tending in bouillon to form short chains. It stains readily with Ziehl's carbol fuchsin, anilin gentian violet, and Loeffler's methylene blue. When stained from 24-hour potato-agar cultures with methylene blue, the cells are 1.7 by 0.6μ with extremes in length from 1.2 to 3.0μ and in width from 0.4 to 1.0μ.

The organism is motile by means of from one to four unipolar flagella which are about 2 to 3 times the length of the cell (fig. 2). Flagella have been demonstrated in agar cultures 1 to 4 days old by the methods of Casares-Gil, of Duckwall, and of Loeffler, as modified by Shunk.

Endospores and marked involution forms have not been noted. With the use of Ribbert's dahlia capsule stain, both the Wisconsin and the eastern strains tested have shown a thin but definite enveloping sheath. The organism is decolorized by Gram's method.

CULTURAL CHARACTERS

The cultural characters as here described were first worked out at Madison with the Wisconsin red-clover organism. It has therefore seemed expedient to designate that as the type strain, and unless otherwise specified the following descriptive characters are based on these Wisconsin studies. In all cases, however, the results obtained in the Raleigh and Washington studies are in essential agreement with these. In the Wisconsin studies the nutrient broths contained 1 per cent peptone and 0.3 per cent Liebig's beef extract; the nutrient agars the same with addition of 1.8 per cent of bacto-agar; the peptone broths either 1 or 2 per cent of Difco peptone. The cultures were kept in dark, well-ventilated incubators held at approximately 25° C. Color determinations follow the Ridgway color standards.[4]

AGAR POURED PLATES.—On nutrient agar, colonies appear after 48 hours, and in 5 days have attained a diameter of 2 to 3 mm. They are circular in outline with entire margins, convex or slightly umbonate, smooth, glistening, and opaque white. Submerged colonies remain small and are lenticular in shape. The agar is unchanged in color and no odor is developed.

On potato agar[5] growth is more abundant, and in 5 days the colonies are 3 to 4 mm. in diameter. They are circular, having entire margins, a surface which is rugose at the center, and a tendency toward contoured markings at the periphery (Pl. 5). The colonies are white and butyrous in consistency.

AGAR STABS.—On nutrient agar the surface growth was at first moderate with a faintly beaded outline along the line of the stab. Later it became more abundant at the surface with colony characters like those on plates. A decided fluorescence was apparent in cultures 2 weeks old.

The growth on potato agar was limited to the upper one-half inch of the line of stab as a faintly beaded line. The surface growth was moderate, convex, smooth, glistening, and opaque grayish white. The colonies become larger than in poured plates but are never larger than 3 to 4 mm.

AGAR SLANTS.—From 5 to 10 days are required on nutrient agar to secure an abundant growth. It is then filiform, spreading, with an entire margin toward the base of the stroke, glistening, and translucent. When one removes a portion of the growth with a platinum needle, it is found to adhere in a butyrous opaque mass. A slight fluorescence appears in the medium in 2-week-old cultures.

On potato agar more abundant growth occurs which is dull and raised. The surface is rugose with radial folds extending outward from the line

[4] RIDGWAY, ROBERT. COLOR STANDARDS AND COLOR NOMENCLATURE. 43 p. 53 col. pl. Washington, D. C. 1912.

[5] Potato agar as used in the Wisconsin laboratory contained 1,000 cc. water, 200 gm. potato, 20 gm. dextrose, and 18 gm. bacto-agar.

of the stroke to the contoured margin (Pl. 6). No odor is developed, and the agar is unchanged.

GELATIN PLATES.—On gelatin plates visible growth was slow, but after five days the colonies were 3 to 4 mm. in diameter with a smooth surface and entire margin. They were finely granular within, especially toward the center. The organism is not capable of liquefying the substratum.

GELATIN STABS.—Best growth occurs at the surface in stab cultures, with neither liquefaction nor discoloration of the medium.

POTATO CYLINDERS.—On steamed potato cylinders growth is first manifest by a faintly yellowish white, spreading streak. It becomes abundant within six to eight days, but remains flat, grayish, and gelatinous in consistency. The cylinder along the line of growth becomes smoky gray. No marked dissolution of the potato tissue occurs, and the potassium iodid test indicates a weak diastasic activity only in 3-week-old cultures.

MILK.—Plain sterilized milk turns creamy in color after two weeks; after five weeks a soft curd forms which slowly separates into whey and a rather firm curd which was not digested at the end of four months. The whey becomes alkaline with litmus as an indicator.

LITMUS MILK.—Lavender-colored litmus milk changes rather rapidly through dark plumbago blue at the end of 5 days, deep Dutch blue at the end of 10 days, to light Tyrian blue at the end of 2 weeks. Curd begins to form soon afterwards and becomes dull tan, whereas the whey is dark blue.

METHYLENE BLUE IN MILK.—Decolorization had been completed in two weeks, but slowly returned with the separation of curd and whey. The color reappeared in the whey.

BLOOD SERUM.—Stroke cultures showed in two to three days' moderate growth, spreading, flat, smooth, and glistening with an echinulate margin. No liquefaction occurred and no discoloration of the medium was noted.

SYNTHETIC MEDIA.—Cohn's solution does not appear to support growth.

In Fermi's solution it soon develops turbidity, and after three days sufficient growth has taken place to produce a milky white cloudiness. After five days a thin viscid pellicle will have formed. The medium gradually takes on a bluish green fluorescence.

In Uschinsky's solution two or three days' growth results in a milky-white cloudiness. Delicate, flocculent pellicle-like growths appear at the surface. The fluorescence which gradually develops is not so marked as in Fermi's solution.

DIGESTION OF CASEIN.—Poured plate cultures in casein agar after a week's incubation had developed colonies 5 to 6 mm. in diameter. When these cultures were tested by flooding with a 1 per cent solution of hydrochloric acid to precipitate the casein, it was found that a narrow zone surrounding each colony remained transparent, whereas the remainder of the plate was milky white. This indicates that the organism is able to digest the casein in the zone immediately surrounding the colonies.

AMMONIA PRODUCTION.—Cultures in peptone broth and beef extract-peptone bouillon 7 days old showed ammonia to be present when tested with Nessler's reagent.

REDUCTION OF NITRATES.—Tests were made with Trommsdorf's reagent in tube cultures containing 2 per cent peptone broth to which 2

per cent potassium nitrate had been added. Satisfactory growth occurred, but no indication of nitrites was secured when the test was applied at the end of 7, 14, and 21 days.

TOLERATION OF SODIUM CHLORID.—Tubes of neutral beef extract-peptone bouillon containing 0.5, 1, 1.5, 2, 3, 4, and 5 per cent of pure sodium chlorid were used in this test. Best growth occurred in the presence of 0.5 per cent sodium chlorid. Higher concentrations of salt were progressively inhibitive, since the growth in 1 and 1.5 per cent was less than in 0.5 per cent, and that in 2 per cent very slight and with no visible clouding in higher concentrations.[6]

GAS PRODUCTION.—These tests were conducted by using fermentation tubes filled with solutions prepared as follows: A 2 per cent peptone solution was used as the base for six solutions made by adding 2 per cent of the following carbon compounds—glycerin, mannite, lactose, maltose, dextrose, and saccharose. These were prepared in sets of 14 each, and were then sterilized and incubated to determine their sterility before inoculation. Eight were then inoculated with Wisconsin type strain 1916–II, three with strain 1919–I, and three with 1920–III. They were not disturbed during the period covered by the test. No gas was produced in any case, and growth was sharply limited to the open arm in all media with each of the strains.

The media for another series were prepared by using a bouillon consisting of 1 per cent Difco peptone, 0.3 per cent Liebig's beef extract, and 0.5 per cent sodium chlorid, as a stock solution. The same carbon compounds were employed and they were prepared separately in 20 per cent solutions in distilled water. These solutions were sterilized at 10 pounds pressure for 10 minutes. The sugars were then added to the bouillon under aseptic conditions and the solutions poured into sterile fermentation tubes. After incubation for 48 hours to determine their freedom from contamination, they were inoculated with strain 1920–III and a single strain from each of three species of clover. Five tubes were used in each set of sugars with each strain. No gas was developed, and no growth took place in the closed arm, results entirely in accord with the previous test.

CARBON METABOLISM [7]

AGAR WITH SUGARS.—Agar was employed in the qualitative studies to determine the production of acid from the several common carbon compounds. It was prepared by adding to flasked and sterilized bacto-agar, cooled to 60° C. and adjusted colorimetrically to P_H 7.4, sufficient of the stock solutions of the carbon compounds to make 1 per cent of the sugar to be tested. Phenol red was added as an indicator. Before the agar had solidified it was poured into sterile test tubes for use in stab cultures. Dextrose, saccharose, lactose, maltose, and glycerin were tested in this manner. After the tubes of media had been incubated sufficiently long to determine freedom from contamination, they were inoculated in sets of five cultures on each sugar with Wisconsin strain 1920–III, and with a strain from red clover, one from white, and one from alsike from Raleigh, N. C. The red color disappeared with all strains in dextrose agar and in saccharose agar within five to seven days, indicating acid production,

[6] At Washington in beef infusion bouillon + 13 Fuller's scale, P_H 6.7, with sodium chlorid added, the Wisconsin as well as the Virginia organisms gave growth in concentrations up to 4 per cent sodium chlorid.

[7] These statements concerning carbon metabolism are based on studies made by F. A. Wolf at Raleigh, N. C. The results with all carbohydrates tested in Wisconsin were in agreement with these.

but all cultures became progressively more alkaline in the other compounds.

BOUILLON WITH SUGARS.—Liquid media were used to follow the progressive changes in hydrion concentration produced during the fermentation of the several carbon compounds. Plain bouillon consisting of 1 per cent Difco peptone, 0.3 per cent Liebig's beef extract, and 0.5 per cent sodium chlorid served as a stock solution. The sugars prepared separately were added to this bouillon to make a concentration of 1 per cent.

During the course of this investigation the organism causing bacterial leafspot of clover has been used in parallel cultural studies with *Bacterium glycineum* and *Bact. sojae* from soybean and found to possess many characters in common with them. These studies as regards the soybean organisms have been reported in other papers (*2, 6, 10*). In view of the fact that it was found to be impossible to separate the clover organism from *Bact. sojae* on the basis of its fermentative ability on media containing the common sugars, it was believed that the "rare" sugars could be employed with success in distinguishing them. Accordingly, in addition to preparing bouillon containing dextrose, saccharose, lactose, maltose, or glycerin in concentrations of 1 per cent, a series of tubes were prepared with bouillon containing 1 per cent of either of the following: The pentoses, xylose and arabinose; the methylpentose, rhamnose; the hexoses, levulose and galactose; the alcohols, mannitol and dulcitol; the polysaccharids, inulin and dextrin; and the glucoside, salicin. Three strains of the pathogen, one from red, another from white, and the third from alsike clover, were used in one of these series. The tubes containing the cultures to be tested were compared colorimetrically with standard buffer solutions. Changes in reaction resultant on fermentation were followed by readings at 24-hour intervals. A considerable number of cultures of each strain with each sugar was employed so that several different tubes could be used in each consecutive reading. The results of these fermentation tests are assembled in Tables I, II, and III.

TABLE I.—*Fermentation of various carbon compounds in plain bouillon by the organism from alsike clover* (initial P_H 7.2)

Carbon compound.	Age of culture and P_H concentration.					
	2 days.	3 days.	4 days.	5 days.	6 days.	7 days.
	P_H	P_H	P_H	P_H	P_H	P_H
Dextrose	7.2	7.0	6.6	6.2	6.4	6.6
Saccharose	7.2	7.2	7.0	6.6	6.2	6.6
Lactose	7.2	7.2	7.2	7.4	7.4	7.6
Maltose	7.2	7.2	7.2	7.2	7.2	7.4
Glycerin	7.2	7.2	7.2	7.4	7.4	7.6
Inulin	7.2	7.4	7.6	7.6	8.0	8.0
Dextrin	7.2	7.2	7.2	7.4	7.4	7.6
Arabinose	7.2	7.2	7.2	7.4	7.4	7.6
Xylose	7.2	7.2	7.2	7.4	7.4	7.6
Levulose	7.2	7.2	7.2	7.4	7.4	7.6
Dulcitol	7.2	7.4	7.4	7.6	7.8	7.8
Mannitol	7.2	7.4	7.4	7.4	7.6	7.6
Salicin	7.2	7.2	7.2	7.2	7.4	7.6
Galactose	7.2	7.2	7.2	7.2	7.2	7.4
Rhamnose	7.2	7.2	7.2	7.4	7.4	7.6

TABLE II.—*Fermentation of various carbon compounds in plain bouillon by the organism from red clover* (initial P_H 7.2)

Carbon compound.	Age of culture and P_H concentration.					
	2 days.	3 days.	4 days.	5 days.	6 days.	7 days.
	P_H	P_H	P_H	P_H	P_H	P_H
Dextrose	7.2	7.2	7.0	6.6	6.2	6.6
Saccharose	7.2	7.0	6.8	6.6	6.6	7.0
Lactose	7.2	7.4	7.4	7.4	7.4	7.6
Maltose	7.2	7.2	7.4	7.4	7.4	7.6
Glycerin	7.2	7.2	7.4	7.6	7.6	7.8
Inulin	7.2	7.4	7.6	7.8	7.8	7.8
Dextrin	7.2	7.4	7.4	7.4	7.4	7.6
Arabinose	7.2	7.2	7.4	7.4	7.4	7.6
Xylose	7.2	7.2	7.4	7.4	7.4	7.6
Levulose	7.2	7.2	7.2	7.2	7.4	7.6
Dulcitol	7.2	7.4	7.4	7.4	7.4	7.6
Mannitol	7.2	7.4	7.4	7.4	7.4	7.6
Salicin	7.2	7.4	7.6	7.6	7.6	7.8
Galactose	7.2	7.2	7.2	7.2	7.4	7.4
Rhamnose	7.2	7.2	7.4	7.4	7.4	7.4

TABLE III.—*Fermentation of various carbon compounds in plain bouillon by the organism from white clover* (initial P_H 7.2)

Carbon compound.	Age of culture and P_H concentration.					
	2 days.	3 days.	4 days.	5 days.	6 days.	7 days.
	P_H	P_H	P_H	P_H	P_H	P_H
Dextrose	7.2	6.6	6.4	6.2	7.2	7.4
Saccharose	7.2	7.0	6.8	6.4	6.4	7.0
Lactose	7.2	7.4	7.6	7.6	7.8	8.0
Maltose	7.2	7.4	7.6	7.6	7.8	7.8
Glycerin	7.2	7.4	7.6	7.8	7.8	8.0
Inulin	7.2	7.4	7.4	7.6	7.8	7.8
Dextrin	7.2	7.2	7.4	7.4	7.6	7.8
Arabinose	7.2	7.2	7.4	7.4	7.6	7.8
Xylose	7.2	7.4	7.4	7.6	7.6	7.8
Levulose	7.2	7.4	7.6	7.6	7.8	8.0
Dulcitol	7.2	7.4	7.4	7.6	7.6	8.0
Mannitol	7.2	7.4	7.6	7.8	8.0	8.0
Salicin	7.2	7.4	7.6	7.8	7.8	7.8
Galactose	7.2	7.2	7.4	7.4	7.6	7.8
Rhamnose	7.2	7.4	7.4	7.4	7.6	7.8

These results so far as the utilization of dextrose and saccharose is concerned confirm those with agar. Furthermore, it is evident that these strains of bacteria represent a single species since they are identical in ability to bring about the hydrolysis of dextrose and saccharose. There is a reversal of reaction with these two carbohydrates due, as has been shown by Wolf and Foster (*11*) with certain other forms, to a lack of sufficient fermentable sugar to permit the attainment of the final maximum hydrion concentration. The clover organism is unable to utilize the other sugars.

After having established the fermentation relations of the clover organism, another series of tests was initiated to determine points of similarity and difference between it and *Bacterium sojae*. In these tests clover strain 1920–III and the soybean organism were grown in comparative cultures with the results shown in table IV.[8]

TABLE IV.—*Comparative carbohydrate fermentations of clover strain 1920–III and Bacterium sojae in bouillon containing 1 per cent sugar (initial P_H 7.4)*

Carbon compound.	Age of cultures, organism, and P_H concentration.					
	3 days—Organism.		5 days—Organism.		7 days—Organism.	
	Clover.	Soybean.	Clover.	Soybean.	Clover.	Soybean.
	P_H	P_H	P_H	P_H	P_H	P_H
Dextrose	7.0	6.8	6.4	6.6	6.8	7.2
Saccharose	7.0	6.8	6.2	6.4	6.6	6.6
Lactose	7.4	7.6	7.6	7.8	7.8	8.0
Maltose	7.4	7.6	7.4	7.6	7.6	7.8
Glycerin	7.4	7.4	7.6	7.6	7.8	7.6
Inulin	7.4	7.4	7.6	7.8	7.8	8.0
Galactose	7.4	7.4	7.6	7.6	7.6	7.6
Arabinose	7.4	7.2	7.6	6.6	7.8	5.8
Xylose	7.4	6.8	7.6	6.0	7.6	5.4
Levulose	7.4	7.2	7.6	7.0	7.6	6.8
Dulcitol	7.6	7.4	7.8	7.6	8.0	7.8
Salicin	7.4	7.4	7.6	7.6	7.8	8.0
Rhamnose	7.4	7.4	7.6	7.6	7.8	8.0

Strain 1920–III is able to ferment dextrose and saccharose alone and is therefore identical in carbon metabolism with the other strains from clover. *Bacterium sojae* is able in addition to hydrolyze arabinose, xylose, and levulose, and on this basis, together with inoculation studies to be reported subsequently, is regarded as specifically distinct from the bacterial leafspot of clover.

It might be pointed out at this point that very little use has hitherto been made of the rare sugars in cultural studies of plant pathogens. This is due in part, no doubt, to the fact that host species with more than a single known bacterial disease are less common than with animals. Rare sugars are recognized as invaluable, however, by investigators of forms pathogenic to animals in the separation of closely related species. Winslow, Kligler, and Rothberg (*3*), for example, have pointed out the significance of xylose and rhamnose fermentations in the colon-typhoid group. They found that xylose can be employed in separating the typhoid and paratyphoid B groups from the dysentery and paratyphoid A groups, while rhamnose furnishes a good basis of distinction between the paratyphoid (A and B) groups, and the typhoid and dysentery bacilli. Koser (*4*) has noted that *Bacillus suipestifer* is unable to utilize the disaccharid trehalose, whereas *B. paratyphosus*, *B. schottmülleri*, and *B. enteriditis* can ferment it with the formation of both acid and gas.

[8] At Washington comparative tests were made with the Virginia and the Wisconsin clover organisms in sugar media. Both fermented dextrose and saccharose, but the Virginia cultures produced less acid than the Wisconsin cultures. Neither of these clover strains fermented lactose, maltose, mannitol, galactose, or glycerin.

Certain other investigations as instanced by those with soybean blights (*6*) and tobacco wildfire and angular leafspot (*11*) have shown the value of rare sugars in differentiating closely related plant pathogenic bacteria. No doubt recognition of this value will be further appreciated as a larger number of bacterial diseases of plants come to be known.

TOLERATION OF ACID AND ALKALI[9]

Peptonized beef broth was adjusted with normal solutions of sodium hydroxid and hydrochloric acid as shown in Table V. Titration was made of each grade of bouillon with phenolphthalein for Fuller's scale value, and the P_H value of each was determined by both the colorimetric and the electrometric methods.

Two stock bouillons were used, one a 0.3 per cent solution of Liebig's beef extract, the other a fresh beef infusion. To each of these was added 1 per cent Difco peptone. All the beef-extract media were from the same stock (+8 Fuller's scale value, P_H value 6.7) and all the beef infusion was from one stock (+22 Fuller's scale value, P_H value 6.4). The initial tests of the media and the inoculations were made on the same day. Inoculations were made from 24-hour-old beef-bouillon cultures, all in vigorous condition, having been grown under favorable conditions for a number of days before transfers were made to the bouillon used for inoculations. The cultures were from the following sources:

VIRGINIA CLOVER.—Strain 3, from white clover, natural infection, September, 1921; strain 4, from red clover, natural infection, August, 1921; strain M, from red clover inoculated with strain 3. Reisolated December, 1921.

WISCONSIN CLOVER.—Cultures from the Wisconsin laboratory, No. 276, isolated 1920, and No. 210, isolated 1916.

BACTERIUM SOJAE.—Strain L, a culture received in November, 1921, from the North Carolina laboratory; strain 8, a reisolation made in December, 1921, at Washington from soybeans inoculated with strain L.

BACTERIUM GLYCINEUM.—No. 270, isolated from soybean in 1919, culture received November, 1921, from the Wisconsin laboratory.

These several cultures of the various organisms had previously been compared with others from the same host plant and had been found entirely representative, and all, except the Wisconsin clover cultures, were actively pathogenic. The Wisconsin cultures were perhaps from earlier isolations and produced in the Washington experiments in 1921 and 1922 only weak infections.

Bouillons for this test were inoculated in February, 1922. The inoculated tubes were kept in a dark, well-ventilated room at 26 to 27° C.

The results of this test as shown in Table V indicate that the Virginia clover strains are less tolerant of alkali than any of the other organisms. The Wisconsin clover strains show only slightly less tolerance of alkali than the soybean organisms. A difference in the age of the Wisconsin and Virginia clover strains may account for their different reaction in alkaline media.

[9] The following statements and table on toleration of acid and alkali are based on studies made by Lucia McCulloch at Washington, D. C.

TABLE V.—*Comparative optimum reaction and toleration limits of the clover bacteria, and Bacterium sojae and Bacterium glycineum.*

ORGANISMS, GROWTH, AND P_H CONCENTRATION ON SEVENTH DAY

Initial tests of media.			Clover, Virginia isolations.						Clover, Wisconsin isolations.				*Bact. sojae.*				*Bact. glycineum.*		Control.	
	P_H reading.		Strain M.		Strain 3.		Strain 4.		No. 276.		No. 210.		Strain L.		Strain 8.		Strain No. 270			
Fuller's scale reading.	Colorimetric.	Electrometric.	Growth.	P_H.	Growth.	P_H.	Growth.	P .	Growth.	P_H.	Growth.	P_H.	Growth.	P_H.	Growth.	P_H.	Growth.	P_H.	Fuller's scale reading.	P_H reading, colorimetric.
BEEF (FRESH INFUSION) BOUILLON WITH 1 PER CENT DIFCO PEPTONE.																				
+30	5.5	5.5	+	6.6	+	6.8	+	6.6	+	6.8	+	5.9	−	.....	−	.....	−	.....	+35	5.5
+27	6.0	5.9	++	7.0	++	7.2	++	6.8	++	7.1	+++	7.1	++	7.2	+++	7.2	+++	7.0	+30	6.0
+22	6.4	6.3	++	7.1	++	7.4	++	7.2	++	7.4	+++	7.2	+++	7.1	+++	7.3	+++	7.3	+24	6.3
+18	6.8	6.62	++	7.4	++	7.7	++	7.6	++	7.6	++	7.8	++	7.8	++	7.6	+++	7.6	+21	6.6
+14	7.0	6.93	++	7.6	++	7.8	++	7.6	+	7.8	++	8.0	++	8.2	++	8.2	++	8.2	+16	7.0
+10	7.2	7.26	++	7.6	++	8.0	+	7.6	+	8.0	+	8.0	++	8.2	++	8.4	++	8.2	+14	7.3
+5	7.7	7.61	+	7.7	+	8.0	+	7.8	+	8.2	+	7.8	+	8.4	+	8.4	++	8.4	+7	7.7
0	8.2	8.05	+	7.8	+	8.2	+	7.8	+	8.4	+	7.9	+	8.4	+	8.4	++	8.4	+4	7.8
−3	8.4	8.32	−	.....	±	.....	−	.....	+	8.4	+	8.2	+	8.4	+	8.4	++	8.4	+2	8.2
−5	8.8	8.63	−	.....	−	.....	−	.....	+	8.4	+	8.4	+	8.4	+	8.4	+	8.4	−1	8.4
−9	9.0	8.85	−	.....	−	.....	−	.....	+	8.6	±	8.4	+	8.6	+	8.4	+	8.4	−4	8.6
BEEF (EXTRACT) BOUILLON WITH 1 PER CENT DIFCO PEPTONE.																				
+28	3.4	3.43	−	.....	−	.....	−	.....	−	.....	−	.....	−	.....	−	.....	−	.....	+29	3.4
+22	3.9	3.84	−	.....	−	.....	−	.....	−	.....	−	.....	−	.....	−	.....	−	.....	+24	3.9
+17	4.4	4.35	−	.....	−	.....	−	.....	−	.....	−	.....	−	.....	−	.....	−	.....	+18	4.3
+12	5.2	5.08	?	5.4	?	5.4	?	5.4	−	5.3	+	5.3	?	5.2	?	5.2	+	5.2	+13	5.2
+8	6.7	6.7	++	7.6	++	7.8	++	7.6	+++	8.0	++	7.8	+++	8.0	++	8.0	+++	8.0	+7	6.7
+3	7.8	7.87	++	7.8	++	8.4	++	8.2	+++	8.4	++	8.2	+++	8.4	++	8.4	+++	8.4	+4	7.7
−1	8.6	8.59	−	.....	−	.....	−	.....	+++	8.8	+	8.4	++	8.8	++	8.8	++	8.8	−1	8.4
−6	9.5	9.09	−	.....	−	.....	−	.....	+	9.0	+	8.8	++	9.1	++	9.0	++	8.8	−4	9.0
−10	10.0	9.39	−	.....	−	.....	−	.....	+	9.0	−	9.0	+	9.1	+	9.0	+	9.0	−7	9.4

− No growth. + Slight growth. ++ Moderate growth. +++ Abundant growth.
± A few tubes showed slight growth.
? A slight clouding in 3 days which disappeared before the seventh day.

In beef infusion media with a P_H value of 5.5 the soybean organisms produced no growth and the clover organisms did not grow until after three to five days and then only slightly. From the delayed and slight amount of growth, it is assumed that in beef infusion a P_H value of 5.5 represents about the limit of acid toleration.

Growth records made on third and fifth days after inoculation show that for all these organisms P_H values 6.4 to 6.7, in either beef infusion or beef extract media, were most favorable for production of early and continued growth.

TEMPERATURE RELATIONS

The organism grows well at a wide range of temperature. In one series of trials at Madison on this phase of the problem cultures on potato-agar slants and in bouillon were incubated at temperatures ranging from 3° to 39° C. at 3° intervals. In another, temperatures of 25°, 30°, 31°, 33°, 34°, 35°, 37°, and 38° C. were maintained. The parasite was able to make appreciable growth at 3° C. Its optimum in bouillon was 26°, whereas in agar 18° to 21° C. appeared to be most favorable. Slow but continued growth could be maintained at 34° C., but 35° inhibited development.

In determining the thermal death point in studies at Madison, two loopfuls of 48-hour-old broth culture were transferred to tubes of beef-extract-peptone broth. After 10-minute exposures in the usual manner the tubes were cooled and incubated. As a result of two series of trials, it was determined that the thermal death point lies between 48° and 49° C.

Experiments at Washington with the Virginia strains gave results entirely in agreement with these statements.

RESISTANCE TO DESICCATION

Vigorous broth cultures, 48 hours old, were diluted with water to twice the orginal volume, and drops of this suspension were allowed to dry on sterile cover glasses kept in sterile Petri dishes. After the drops had dried, tests were made by inserting the cover glasses into tubes of nutrient broth. No growth appeared after 30 minutes' desiccation, so that the organism is to be regarded as very susceptible to drying.

Tests in Washington with the Virginia strains gave the same results.

VITALITY ON CULTURE MEDIA

The clover organism has been found to retain a vitality on the usual culture media for an indefinite period, having been maintained in Wisconsin on potato agar without appreciable loss of vigor for about four years.

PATHOGENICITY

The type organism from red clover in Wisconsin has been found to hold its pathogenicity in agar culture for about one year but has tended gradually to lose it thereafter. The strains from the other localities have behaved similarly. The preliminary pathogenicity trials made at Madison, Wis., in 1916 included inoculations of red clover and soybeans, since it was then thought that the bacterial leafspot and soybean blight

might be indentical. Infections were readily secured upon red clover, but the inoculations of soybeans were unsuccessful. Other isolations including strain 1919–I, made in the summer of 1919, were successfully employed in infecting red clover in another series of experiments. Again, in 1920, a more extended series of tests of pathogenicity was instituted. These trials included the four clover species, *Trifolium pratense*, *T. medium*, *T. repens*, *T. hybridum*, and the white sweet clover, *Melilotus alba*. Four recently isolated strains of the organism, three from *Trifolium pratense* and one from *T. medium*, were used in these series. Water suspensions of 3- to 4-day-old potato-agar cultures were applied with an atomizer, after which the wetted leaflets were gently rubbed between the fingers. The inoculated plants were then covered from 48 to 72 hours with bell jars when the sky was clear, and were left uncovered if cloudy weather prevailed. Inoculated plants were sprayed once daily with sterile water to favor infection. Plants both out of doors and in the greenhouse were thus inoculated. After a period of incubation of 6 to 10 days, lesions appeared uniformly on all inoculated plants of *Trifolium pratense* and *T. medium*. Inoculated white clover, alsike clover, and white sweet clover, however, remained free from infection. The failures to secure infection of these species receive support from the field observations made in Wisconsin, since no cases of natural infection of these species or of alfalfa have been discovered, although all have been found growing closely intermingled with diseased red clover.

Several series of inoculation experiments have also been conducted at Raleigh, N. C., during the several seasons in which the investigations have been in progress. One of these, in the summer of 1922, is representative in all respects of the others and is, therefore, briefly described. Cultures from each of the clovers, red, white, and alsike, which had been grown for 48 hours on bacto-agar were used as an inoculum. The growth was washed off with sterile water and the bacterial suspension poured into Petri dishes. On the morning of July 22 inoculations were effected by immersing the leaves in these suspensions. The strain from any one of the host species was used to inoculate plants of that species and also the other two species. All inoculated plants were grown in the greenhouse and after inoculation were shaded lightly for 48 hours with newspapers. On July 29 small translucent areas, evident only on the lower leaf surface, were present in abundance on all inoculated plants. Three days later these lesions had developed into small, blackish brown spots characteristic of the disease in nature.

The same strains were also used in North Carolina to inoculate plants of soybean, but no evidence of infection appeared.

At Washington both greenhouse and out-of-door plants were used in the inoculation experiments, which extended through two years. Bacteria from 1- to 4-day-old-agar cultures were washed off in sterile water and this bacterial suspension was then sprayed on the plants with an atomizer. Usually the plants were covered with bell jars or placed in moist chambers for 24 to 48 hours after inoculation, then returned to normal conditions.

The first evidence of infection was noted in from 6 to 10 days as tiny, translucent areas which enlarged in a few days into the nearly black spots with translucent borders. Successful but slower infections resulted if the plants were not kept unusually moist for a period following inoculation. Check plants invariably remained free from infection.

Infected plants continued to develop new infections, but there seemed to be little or no spread of the infection to other plants not in direct contact with infected leaves.

All of the strains isolated at Washington produced infections not only on the original host species but also on other species, and in the numerous cross inoculations no difference was observed in the degree of pathogenicity on the various species. Strains from either red, white, alsike, or other species produced the characteristic lesions on any of the species. The following clovers were artificially infected: *Trifolium pratense* L., *T. pratense* var. *perenne* Host., *T. repens* L., *T. repens* var. *latum* McCarthy, *T. medium* L., *T. hybridum* L., *T. incarnatum* L., and *T. alexandrinum* L.

Lima bean (*Phaseolus lunatus*) and velvet bean (*Stizolobium deeringianum*) were also infected by the Virginia strains of the clover bacteria. On lima beans the infection was slight. The lesions were small, reddish, with a surrounding white zone and without any translucent tissue. On velvet bean the infection was moderate, lesions dark, almost black, circular to angular, and with translucent borders.

At Washington many attempts were made to infect soybeans (*Glycine hispida*). Three varieties—Ito San, Wilson Fine, and Black Eyebrow—were used in the tests. These were inoculated at various ages and with various strains of the clover bacterium, but no infections were ever secured. Similar plants used as controls were readily and typically infected with *Bact. sojae* and *Bact. glycineum*. The number and the thoroughness of these experiments on soybeans give evidence of definite specific differences between the clover and the soybean bacteria.

Two attempts to infect alfalfa gave negative results.

Further inoculation experiments will be necessary to determine how to interpret the differences in pathogenicity observed in the Mississippi Valley and the two seaboard stations. It may be that we are dealing with specialized races, the one restricted to the red clovers, the other having wider host range. It will be recalled that the Wisconsin strain 1920-III which was pathogenic to the red clovers in Wisconsin in the 1920 trial was found in 1921 in North Carolina to be culturally indistinguishable from the strains isolated in that State. It had, however, then lost its virulence even for red clover, indicating that pathogenicity is not a fixed and constant character with this species.

The results at all three stations are, however, in full accord so far as concerns the nonpathogenicity on soybeans. These failures to infect soybean with bacterial leafspot of clover furthermore substantiate the cultural studies in showing that the clover organism and the one from soybean are specifically distinct.

In the Washington laboratory comparative cultural tests, made with the Wisconsin and the Virginia clover strains, indicate that they are not identical in cultural characters. The tests were repeated several times and always gave the same results. These variations may be inherent or merely due to differing ages of culture. The chief cultural differences observed in parallel tests at Washington are summarized below.

VIRGINIA CLOVER BACTERIA.

1. Growth pure white and opaque on agar.
2. Growth extremely viscid on beef media.
3. No fluorescence in beef media.
4. Milk not coagulated. Remains opaque and cream color.
5. Strong reduction of litmus in milk.
6. Congo red in media containing dextrose is unchanged.
7. Potato agar, smooth growth.
8. Potato cylinders, slight growth.
9. In Uschinsky's solution, viscid pellicles and sediment and very slight fluorescence.
10. Casein not digested.
11. Approximate alkali limit, P_H 8.2 to 8.6.

WISCONSIN CLOVER BACTERIA.

1. Growth grayish white and not entirely opaque on agar.
2. Growth butyrous on beef media.
3. Fluorescent in beef media.
4. Milk coagulated. Becomes translucent and brownish.
5. Slight to no reduction of litmus in milk.
6. Congo red in media containing dextrose changed to dark purple-brown (acid reaction).
7. Potato agar, usually contoured growth.
8. Potato cylinders, abundant growth.
9. In Uschinsky's solution, pellicles and sediment not viscid, moderate flourescence.
10. Casein digested.
11. Approximate alkali limit, P_H 9.0 to 9.5.

TECHNICAL DESCRIPTION

Bacterium trifoliorum, n. sp.[16]

Cylindrical rods rounded at ends, solitary or in short chains; cells 1.2 to 3.0 μ by 0.4 o 1.0 μ, with an average length of 1.7 μ and a width of 0.7 μ; motile by means of one o four unipolar flagella; aerobic, Gram negative, no spores; not conspicuously apsulated.

Colonies on nutrient agar grayish white, glistening, margins entire, convex or lightly umbonate.

Gelatin not liquefied, nitrates not reduced, digests starch very feebly, acid formed rom dextrose and saccharose, no gas produced in various carbohydrate media.

Group number 212.2322023, following the descriptive chart of 1917 of the Society of American Bacteriologists.

Type collected at Madison, Wisconsin, on *Trifolium pratense* L.

The type strain from Wisconsin has proved pathogenicity only on the red clovers *Trifolium pratense* L. and *T. medium* L.); the strains from North Carolina and the vicinity of Washington, D. C., otherwise scarcely distinguishable, have been proved pathogenic also on *T. repens* L., *T. hybridum*, L., *T. incarnatum* L., *T. pannonicum* L., and *T. alexandrinum* L. Lesions occur on leaves, stems, petioles, stipules, and flowers.

Distribution apparently widespread in the northern Mississippi Valley on the red clovers *T. pratense* and *T. medium*, and the same organism, at least on the Atlantic seaboard, occurs on *T. repens*, *T. hybridum*, *T. incarnatum*, *T. pannonicum* and *T. alexandrinum*.

Specimens on all these hosts showing both natural and artificial infections have been deposited in the herbaria of the department of plant pathology, University of Wisconsin, and the Bureau of Plant Industry, United States Department of Agriculture.

RELATION OF PARASITE TO HOST TISSUE

Infection apparently occurs through the stomates. This has been evidenced by noting that lesions first appear on the lower leaf surface, the one occupied by the breathing pores. Then, too, young lesions have been fixed in alcohol, embedded, sectioned, and stained with methylene blue. The intercellular spaces of the mesophyll immediately subjacent to the stomates in such sections are seen to be densely packed with bacteria. In older lesions in which the host cells have collapsed, the parasite may invade the cell cavities.

[16] According to the classification of Migula and the recent report of the committee of the Society of American Bacteriologists the name of this clover organism would be *Pseudomonas trifoliorum*, n. sp.

OVERWINTERING AND DISSEMINATION

No experimental evidence on the overwintering and dissemination of *Bacterium trifoliorum* is at hand. The field observations, however, during the several seasons in which the disease has been investigated show that it recurs annually in the same general areas in the clover fields and on the same small groups of plants in meadows and lawns. One old clover field at Madison was observed almost daily during April, May, and June, 1920. The disease appeared, with the advent of warm weather in April, upon the young leaves soon after they had unfolded. Here, no doubt, fallen diseased leaves harbored the parasite during winter and it spread from them to the new leaves. The disease was very much in evidence during May, and by blossoming time every leaf on a plant might be conspicuously spotted.

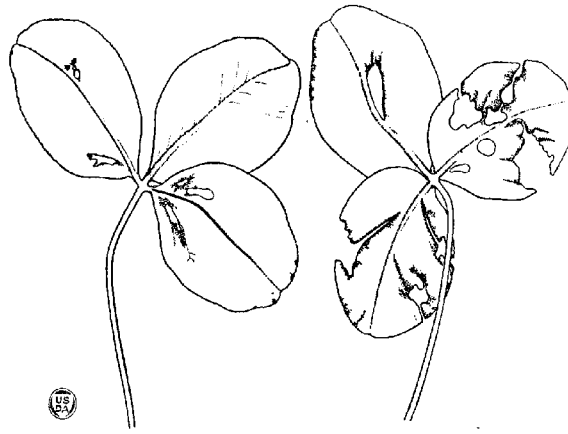

FIG. 3.—Diagram of two clover leaves in which insect injuries (*Phytonomus punctatus*) and the bacterial lesions (*Bacterium trifoliorum*) are associated. In the leaf at the left, the lesions were young and water-soaked and apparently had originated at the insect puncture. In the leaf at the right, the insects may, at least in some places, have eaten out the invaded tissue. In any case, the water-soaked margins indicated that the invasion was still progressing. (Drawing by Charles Drechsler.)

The fact that the disease under favorable conditions involves the plant so generally makes it highly probable that the floral parts might become infected. Lesions have not been observed, however, on the floral organs, but they are of common occurrence on the flower pedicels. Even though flowers are not involved, there would be ample opportunity for the seed to become contaminated either while yet in the field or during harvesting or threshing. The initial infections in newly planted fields could thus come from contaminated seed. That seed serves as the primary source of infection is indicated by the occurrence of diseased plants in newly planted pastures and lawns. Should this disease ever become seriously destructive, any precautionary measures looking toward its control or prevention, especially in new plantings, should stress the possible value of seed disinfection.

The most rapid spread of bacterial spot, as shown by field observations, occurs when there is an abundance of rain or dew. At these times conditions are most favorable for the spattering of the bacterial exudate to adjacent healthy leaves of plants, and opportunity is given for the bacteria to gain entrance to the leaves by means of the surface film of moisture.

Aside from rain and dew as agents of dissemination of bacterial leafspot, there is considerable observational evidence that certain leaf-eating insects, especially the larvae of the clover leaf weevil, *Phytonomus punctatus*, are responsible for its spread. Initial infections not uncommonly occur at the places where the leaves are injured by the feeding of these insects. In experimental feeding trials, clover leaf weevils avoided in every case eating other than the healthy tissues of abundantly spotted leaves offered them. The fact, however, that perforations made by weevils are the loci of infection indicates insect carriage. Many other plant pathogens whose normal mode of entering is through stomates are known to gain entrance also through wounds.

SUMMARY

(1) A hitherto undescribed bacterial leafspot disease has been observed on several species of clover, including *Trifolium pratense*, *T. medium*, *T. repens*, *T. repens* var. *latum*, *T. hybridum*, *T. incarnatum*, *T. alexandrinum*, and *T. pannonicum*. It is known to occur in Wisconsin, Iowa, Indiana, Virginia, Maryland, and North Carolina, and is probably widely prevalent.

(2) Leaves, stems, stipules, petioles, and flower pedicels are known to be involved, but lesions have not been observed on the floral organs.

(3) The spots may appear at any time throughout the growing season. The lesions on the leaves are at first minute, translucent dots which enlarge and become, at length, irregular, blackish-brown areas. These areas have a translucent border and the surrounding tissues are yellowish green. Mature leaves are perforated and frayed, due to the drying and falling of portions of the affected tissues.

(4) Under favorable moisture conditions, a milky white bacterial exudate is formed on the lower leaf surface. On drying, this becomes a delicate incrusting film.

(5) Bacterial leafspot is caused by an organism which is herein described as *Bacterium trifoliorum*, n. sp. It forms whitish colonies on nutrient agar, is flagellate, and forms acid from dextrose and saccharose. According to the Descriptive Chart, its group number is 212.2322023.

(6) With the type strain from Wisconsin, infection was secured only on the red clovers, but with the strains from North Carolina successful reciprocal inoculations have been made on the red, white, and alsike clovers. The Virginia strains also cross-infect successfully. The parasite is intercellular and apparently enters chiefly through the stomates.

(7) Field observations indicate that the dissemination of the disease is accomplished through the agency of rain or dew and of leaf-eating insects.

(8) It seems very probable that the organism is disseminated with the seed and that such contaminated seed in new plantings are in consequence the primary loci of infection.

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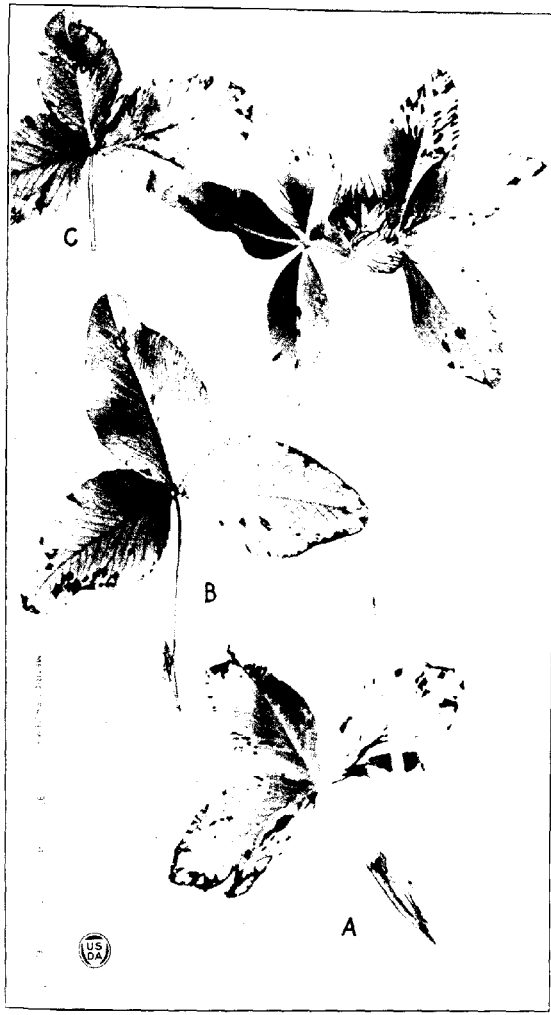
PLATE 1

Bacterial leafspot of red clover.

Blighted leaves of red clover (*Trifolium pratense*) from Madison, Wis., showing natural infection in various stages of development, collected by M. M. Williamson at Madison, Wis. Young lesions are characteristically distributed in the upper leaflets of branch A as well as in those of leaf B, while older lesions appear in the lower leaf of branch A and in leaf C. Note the uniform black color of the young lesions as contrasted with those in leaf C, in which the centers are dried out and lighter in color (cf. Pl. 2). The splitting of the dead areas following the drying out of marginal lesions, which causes a ragged appearance of the leaves, is well shown in the lower leaves of branch A and in leaf C.


C
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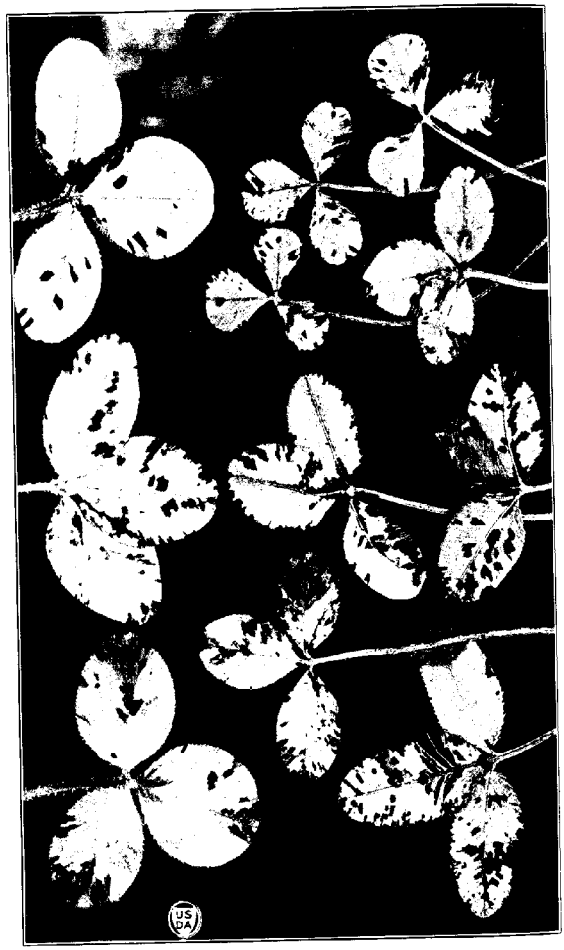


PLATE 2

Bacterial leafspot on the three common clovers for comparison.

The disease is shown as it occurs on the three common clover hosts, natural infection, collected by F. A. Wolf at Raleigh, N. C. The three leaves in vertical alignment at the left are the red clover, *Trifolium prātense*. In the upper right-hand corner are grouped four leaves of the white clover, *Trifolium repens*. The remaining four, at the lower right hand, are the alsike clover, *Trifolium hybridum*. All natural infections.

PLATE 3

Bacterial leafspot of clovers.

A.—*Trifolium medium*, zigzag clover.

B.—*Trifolium repens*, variety *latum*, also known as Ladino clover. Photographed by transmitted light to show the translucent borders surrounding the lesions.

C and D.—Leaf and stipules of *Trifolium pannonicum*. The black streaks in the stipules are due to the bacterial infection.

Collected at Arlington, Va., by Lucia McCulloch. All natural infections. All natural-size photographs.


A
B
C
D
USDA

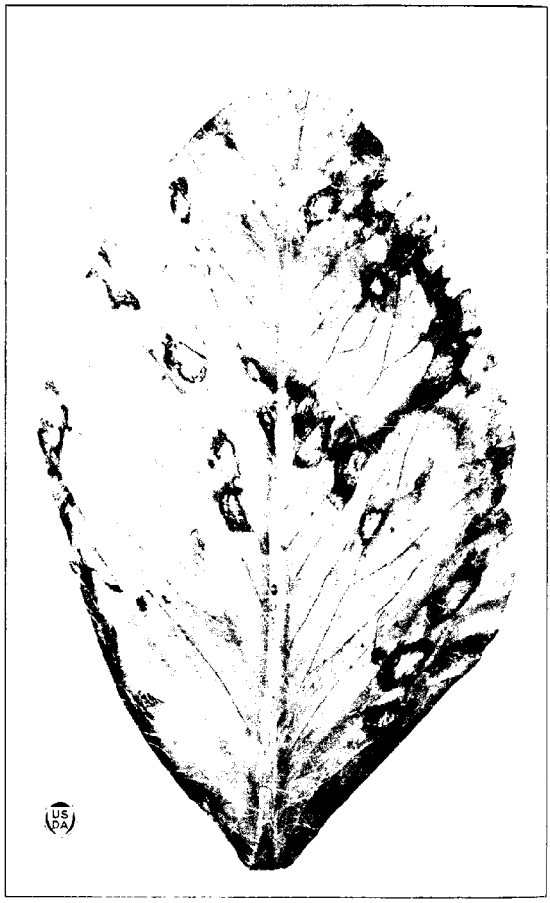


PLATE 4

Diseased leaflet of red clover, magnified to show details.

Photograph of the lower surface of a diseased red clover leaflet, magnified fivefold. This is the central leaflet shown in Plate 1, C. It illustrates the distribution of lesions in the intervenous tissue, also the lighter dried-out central area with dark, water-soaked margin, characteristic of the well-advanced lesion.

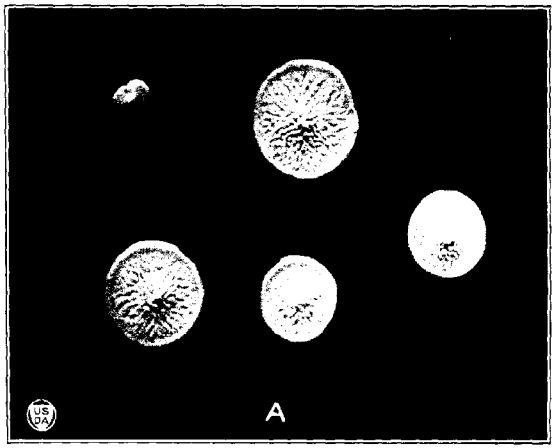
PLATE 5

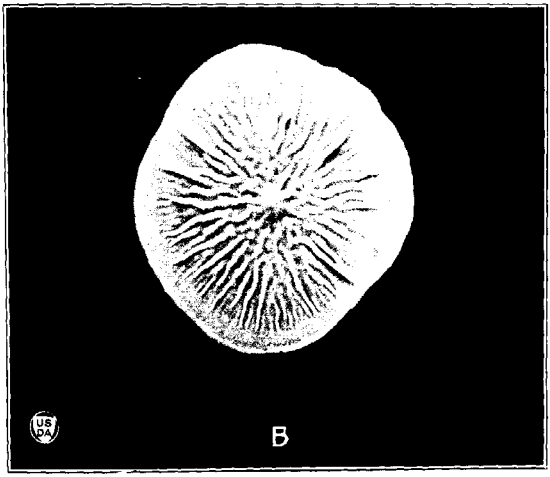
Colonies of the clover organism.

Potato agar plate colonies of *Bacterium trifoliorum* incubated for 9 days at 26° C.

A.—Wisconsin isolation 1916–II, enlarged ×1½, showing circular surface colonies and lens-shaped deep colonies. Note the rugose umbonate centers with radial corrugations extending outward toward the plain marginal zone. In very slow-growing colonies, these radial corrugations are commonly crossed by concentric folds. The plain zone at the margin is commonly raised, although less pronounced in some cases, as shown in B.

B.—Wisconsin isolation 1920–III enlarged ×1½. The same colony characteristics appear in this strain as in 1916–II and other slower growing isolations. Indeed, the amount of growth may not be much greater, for the colonies seem more spreading and relatively thinner. Note, for example, the absence in colony B of the raised edge which is found in those of A. If this strain had not evidenced this spreading characteristic so constantly both in plates and tubes (Pl. 6) of the different agars used, it would seem to be an accident of moisture content. In their reactions in milk, sugar media, etc., the two strains, 1916–II (A) and 1920–III (B), seem identical; therefore these differences are attributed to variations in growth vigor and other minor or transient characters.


A


B

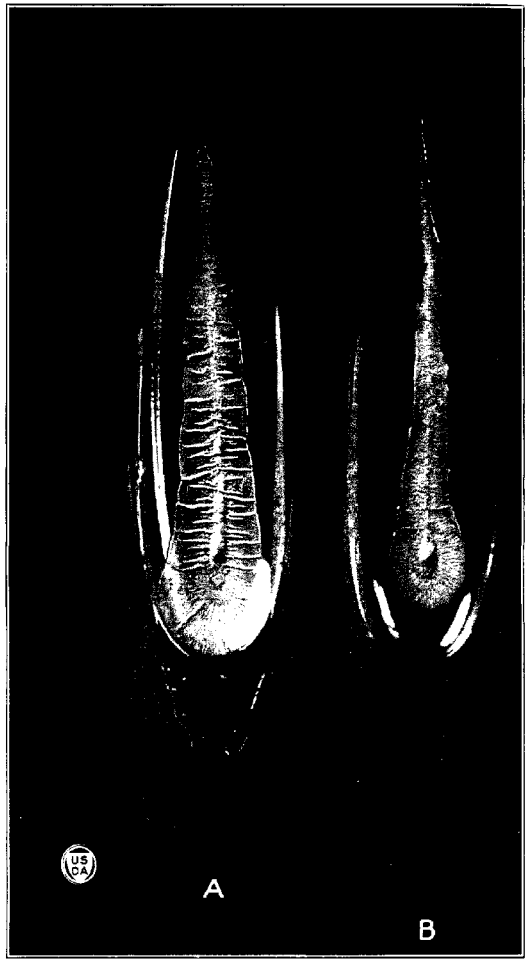

A
B

PLATE 6

Agar slant cultures of the clover organism.

Potato agar (1.8 per cent agar) streaks of *Bacterium trifoliorum* incubated for four days at 26° C.

A.—Wisconsin isolation 1920–III illustrates well the characteristics of this more spreading strain. As in the plate colonies (see B, Pl. 5) the radial folds are not so closely packed, the edges of the colony less raised than in isolation 1916–II shown in B.

B.—Wisconsin isolation 1916–II. (Compare also with colonies in Pl. 5, A.)

A NEW TYPE OF ORANGE-RUST ON BLACKBERRY[1]

By B. O. DODGE

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The writer in discussing the distribution of the orange-rusts of Rubus[2] referred to certain blackberries obtained at Forestville, Md., in 1922. Some of the plants were infected with the typical short-cycled form, one plant with the long-cycled Gymnoconia, and two plants with a form whose spores were reddish orange and corresponded in other particulars to aecidiospores of the long-cycled rust. When spores from these two particular plants were sowed on agar, they germinated so quickly and with such long germ tubes that the rust was at first marked as long cycled. Examination of the plates a day or two later, however, disclosed large numbers of long promycelia bearing sporidia. On repeating the germination test, the same results were obtained. Any long germ tubes that persisted were ignored, or assumed to be abnormal. The presence of hundreds of promycelia could not be overlooked. The appearance of the rust being so like that of the ordinary Gymnoconia and the manner of spore germination so puzzling, a more critical study of this strain of the rust was made in 1923, when the plants were in far better condition for spore production than they had been just after being transplanted the previous year. The results of the later investigation show that three plants are infected with what might be called an intermediate form, because some aecidia are short cycled and others long cycled.[3] It is interesting to find here an example which can be best described as a short-cycled rust being derived from a long cycled form. On April 11, 1922, 12 infected wild blackberries, No. 288 299, were dug up in a pasture at Forestville, Md.; two plants, No. 290 and 294, died soon afterwards. A brief account of this collection of rusted plants may serve to bring out more clearly the true nature of our Rubus orange-rusts.

If one plant is infected with the long-cycled rust, and another with the short-cycled, aecidia will usually, other conditions being equal, mature about a week or 10 days earlier on the latter plant. After the 10 surviving plants in this collection were brought to the greenhouse from the cold frames, March 10, 1923, the date of the maturing of the first aecidia on each was noted. Within three weeks aecidia had matured on six plants. The aecidia on five plants were yellowish orange in color, and their spores produced regular promycelia. This is certainly our common short-cycled blackberry rust. The rust on No. 297 needs further study. About 10 days later aecidia matured on the other four plants. Germination tests were made of spores from several leaves from No. 291 and no promycelium was found. The rust on this plant is typically long-cycled, and is of no particular interest in itself.

It was not until we had improved our methods of testing spore germination that we discovered that the rust on the other three plants was

[1] Accepted for publication June 25, 1923.

[2] DODGE, B. O. THE DISTRIBUTION OF THE ORANGE-RUSTS OF RUBUS. *In* Phytopathology, v. 13, p. 61–74. 1923. Literature cited, p. 74.

[3] For convenience may we not refer to an aecidium as "short cycled" if its spores produce promycelia, and as "long cycled" if they produce germ tubes on germination?

producing two sorts of aecidia. Spores will germinate normally if floated on water or placed in hanging drops, but agar plates are far more convenient and satisfactory for this work. The writer's method now consists in placing a leaf bearing young aecidia on agar in Petri dishes so that spores from individual sori fall together on the surface of the agar as they are naturally shed. A spore print is obtained in a few hours, showing the location of the ruptured sori on the leaf. The same leaf can be used several times to obtain additional spore prints for study. The strongly negatively heliotropic reaction which sends the germ tubes down into the agar will be avoided if the plates are more equally illuminated from below and above. The temperature factor need not be considered in this connection so long as the spores germinate.

A SHORT-CYCLED RUST IN THE MAKING?

As soon as spore prints of individual sori on leaves from plants No. 292, 293, and 296 had been obtained, it was clear that the spores from all sori had not been discharged in the same way. Certain aecidia had shed spores that were very dry or powdery, so that they had been evenly scattered like dust on the agar. The spores from other aecidia seemed to be more waxy and tended to cling together, falling in little clumps, like waxy pollen. The dusty spores were shed from the sorus as soon as mature; the waxy spores tended to stay in the sorus and pile up in irregular masses. It was found that the spores in the waxy sori produced promycelia, while the more dusty spores produced only long germ tubes. Contrary to what one would expect, the waxy sori (short cycled) were of a more reddish orange, while the others (long cycled) were more of a golden orange. The color contrasts showed very distinctly in the spore prints. Considering, then, the waxy nature and the color of the spores, it was not at all difficult to tell by examining a sorus with a hand lens how its spores would germinate.

DISTRIBUTION OF AECIDIA ON THE LEAVES

The leaves on these blackberries have three leaflets. Sometimes all aecidia on the three leaflets will be long cycled, while those on another leaf will be short cycled. Again, all sori on the terminal leaflet will be short cycled, while those on the other two are long cycled. It was frequently noted that aecidia at the base of a leaf or leaflet were long cycled and those toward the tips short cycled, but no general rule seems to be followed in this distribution. Occasionally, a long aecidium was found in which the spores at one end were long cycled and those at the other were short cycled. This might have been caused by the running together of separate sori. The writer has had in mind the task of infecting the same blackberry systemically with both the long-cycled and the short-cycled forms of orange rust. Such an experiment would probably necessitate the development of teleutospores of the Gymnoconia in the greenhouse so that they could be sowed on new shoots of the blackberry at the same time that the aecidiospores of the short-cycled rust were being matured in nature. This would mean that the greenhouse work should begin about six weeks before the orange rust appears in the field. Local gametophytic infections could be affected so that the mycelium of each type would remain isolated in different nodes, to run together at some common node. Should the two mycelia come together in a leaf,

conditions would be right for hybridization. It was at first thought that such a type of infection had actually occurred in nature, thus accounting for the two kinds of sori in the three plants mentioned. The evidence, however, is against such a supposition. The writer has recently obtained a number of blackberries not only from Forestville, but from other localities, in which the rust was maturing two kinds of aecidia. The typical short-cycled rust has waxy, yellowish orange spores, but the short-cycled sori in plant No. 292, for example, are of an even darker and more reddish orange than are the long-cycled sori from the same leaves, and the spores also correspond in shape and size to spores of the typical long-cycled form. If we had two mycelia in the leaf the short-cycled *Caeoma nitens* type should be yellowish orange. The chances that teleutospores of the Gymnoconia and aecidiospores of the short-cycled form would mature at the same time in a given locality are rather remote. The writer has, however, collected good orange-rust aecidia in September. The seasonal conditions which would ordinarily bring out the orange-rust stage of one form would be just as favorable for the development of that stage of the other form. The time intervening between the sowing of the aecidiospores of the Gymnoconia and the development of its teleutospores is at least one month, and is usually much longer. Aside from the old doctrine of immutability of species, the best evidence that two mycelia are actually present in these leaves is the fact that the two types of aecidiospores are generally borne in separate sori.

Several blackberries have been inoculated with aecidiospores from No. 292, 293 and 296. Teleutospores have already matured. We shall wait with interest until next spring to learn, in case there is systemic infection, whether or not the new rust will develop two sorts of aecidia. If it does it will have been proved that this phenomenal development of two kinds of aecidia is not due to the presence of two different orange-rust mycelia in the same leaf, but is due rather to the unstable nature of this particular form.

The writer stated in a recent paper [4] that if one should visit Schweinitz's old collecting ground at Salem, N. C., about May 15, he would probably find the long-cycled Gymnoconia on blackberry. The opportunity was afforded the writer himself to make this trip May 19, 1923, and he had no difficulty in picking up any number of specimens of rust on blackberry which by color and test by spore germination were proved to be long cycled. Just which rust Schweinitz [5] had, however, when he described *Caeoma nitens* is far from a certainty. The Gymnoconia was also found to be very common at Cornelia, Ga., May 17. Teleutospores will be developed on blackberry here about August.

The short-cycled rust on dewberry in more southern areas is a well-fixed form, morphologically distinct from the Gymnoconia on black raspberries and on *Rubus saxatilis*, of Europe and Asia. The fact that one form is maturing two kinds of aecidia, as described above, may indicate that a third type is arising. The aecidiospores are far more like those of the long-cycled Gymnoconia. The evidence based on distribution also favors the belief that the long-cycled orange-rust is the more primitive. Only this type occurs in Europe and Asia, and it is now known over most of North America, probably extending as far south and west as the short-cycled form. The westerly winds might well have

[4] DODGE, B. O. OP. CIT.
[5] SCHWEINITZ, Lewis David von. SYNOPSIS FUNGORUM CAROLINAE SUPERIORIS . . . ed. a D. F. SCHWAERICHEN. 105 p., 1 col. pl. 1822. E. Commentariis, Societates naturae curiosorium lipsiensis excerpta.

spread the Gymnoconia, with its somewhat dry and dusty spores, from western Europe through Russia into Siberia and across the straits to Alaska. From there it probably followed the coast and crossed the continent from west to east and from north to south. Many additional susceptible species of host were met in America, at the same time that widely different climatic conditions were encountered, all of which favored a change of life habits by the rust, which culminated in the elimination of the teleutosporic stage entirely. Until some one has secured systemic infection of blackberry directly by sowing aecidiospores from the raspberry, which Kunkel says sometimes produce promycelia, and has obtained a systemic rust at once comparable to our common short-cycled rust, the writer sees no valid reason why separate specific or even generic names might not be applied to two forms which are well fixed. The third or intermediate type which is maturing aecidia of two kinds may represent a strain of Gymnoconia which is particularly unstable and from which a short-cycled rust is now arising and which will have distinct morphological characters of its own.

As the writer understands Kunkel's [6] conception of the two life cycles involved in the Gymnoconia, the aecidiospores are all alike in that, especially if germination can be delayed by lowering the temperature, a few normal as well as abnormal promycelia are apt to be found in almost any germination test. This may very well be the true nature of the common Gymnoconia, but the form of the rust which produces two kinds of aecidia is in an entirely different category. In this form the nature of the aecidiospore, whether it is to produce a germ tube or a promycelium, is predetermined as it is being matured. The fusion in the spore of its two nuclei might very well be accompanied by changes in the coloration of the spore contents and in the nature of the spore wall, but by no kind of treatment during germination could a normal aecidiospore germ tube be obtained. On the other hand, precocious nuclear fusion, either in the spore or in its tube, might be induced by some external treatment so that a promycelium with sporidia would follow. In case such sporidia were capable of infecting the host, we should expect the rust to go back to its former habits in the new generation resulting from this infection. The nature of the germ plasm is believed not to be easily and permanently altered by environmental changes.

The writer has given reasons for believing that the production here of two kinds of aecidia on the same leaf is due to the variable nature of a strain of the Gymnoconia which normally includes the old *Puccinia peckiana* in its life cycle.

Whatever may be one's view regarding the manner of the origin of a species, and whether in this case we say a species is arising as the result of hybridization or by mutation, it is clear that the practice of applying the terms telium and teliospore to the aecidium and aecidiospore of a short-cycled rust just because the aecidiospore happens to produce a promycelium instead of a germ tube can no longer be defended. Such terms should represent morphological units, and not behavior. No doubt a study of the individual aecidia of a number of other rusts which were shown by Jackson at the Boston meeting of the Botanical Society of America to have correlated short and long cycled forms will bring forward additional evidence to prove that what we are pleased to call species are arising to-day as they have arisen for ages past.

[6] KUNKEL, L. O. FURTHER DATA ON THE ORANGE-RUSTS OF RUBUS. *In* Jour. Agr. Research, v. 12, p. 501–512, pl. D, 92–94. 1920. Literature cited, p. 512.

EFFECT OF THE ORANGE-RUSTS OF RUBUS ON THE DEVELOPMENT AND DISTRIBUTION OF STOMATA[1]

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In order to obtain a supply of teleutospores with which to carry on some experiments on infecting the black raspberry (*Rubus occidentalis*) with the gametophytic stage of Gymnoconia, the writer has frequently sowed aecidiospores when the topmost leaves of new canes were still folded, while the lower leaves and all those on old canes were fully expanded. Although many trials, both in the field and in the greenhouse, have resulted in failure to obtain infection, the most abundant production of teleutospores followed inoculation of leaves of the latter type. It has been shown repeatedly that aecidiospore germ tubes gain entrance into a leaf through the stomatal openings. Clinton[2] reports that the stomata of leaves of blackberries and raspberries are confined to the lower surface except for a few which occur along the margin on the upper side. He believes, therefore, that infection by aecidiospores of the orange-rust must occur at a time when the side bearing the stomata is exposed to receive the spores falling from above, which would be when the leaves are beginning to unfold. The ventral surface is then turned outward and somewhat upward, and the halves are so folded together as to expose the margins perfectly to catch the spores. As the results of our experiments did not strongly support the theory that leaves are the most readily infected before they unfold completely, a study was made of the development and distribution of stomata on various types of leaves from blackberries and raspberries. The fact that agitation by the wind and the work of insects might well carry spores to the underside of fully expanded leaves would not minimize the importance of the distribution of stomata in facilitating infection. In the course of this work it was found that the invasion of leaves by the orange-rust mycelium has a very unusual effect on the production of stomata.

If account is taken of the conditions of the leaves of our blackberry or raspberry in nature at the time aecidiospores are being shed, it will be seen that several types are exposed to infection. The first leaves to unfold are the ones on the normal old canes; the infected old canes are also developing their leaves at this time. Practically all leaves on the old canes, normal and infected, are of about the same age. New shoots from the base of infected and of uninfected plants grow up a little later. During the growth period of the new canes there will always be leaves just unfolding at the top, and others lower down fully expanded.

In general, most of the stomata on normal leaves of Rubus are on the lower side, as stated by Clinton. A number of species of this genus have a few on the upper side at the tips of the serrations. Some blackberry leaves examined have a small number irregularly scattered, singly or in groups of two or three, on the upper side, especially along the larger

[1] Accepted for publication June 25, 1923.

[2] CLINTON, G. P. ORANGE RUST OF RASPBERRY AND BLACKBERRY. Ill. Agr. Exp. Sta. Bul. 29, p. 273-300, 4 pl. 1893. Literature, p. 292-295.

veins; in every such case the number is so small as to be of little consequence in the economy of the normal plant. It is quite otherwise in case of plants systemically infected with either of the orange-rusts.

Sections of leaves collected at random for another purpose from a systemically infected blackberry in New Hampshire show fully twice as many stomata on the upper side as they do on the lower side. An examination of sections of black raspberry leaves which had been prepared for a study of young aecidia revealed an abundance of stomata on the upper side. Further investigations disclosed the fact that the systemic stage of these orange rusts regularly so affects the host as to lead to the development of a large number of stomata on the upper side of the leaf where normally there would have been only a very few or none at all. One frequently sees that in a blackberry which has harbored the rust for two years or more most of the leaves on the old canes infected are evenly covered with spermogonia from the time they begin to unfold. From one-fourth to one-half of the total number of stomata on such leaves will be found on the upper side. Certain specimens of infected mountain blackberry did not show such a large percentage of stomata on the dorsal side; the increase in the number due to the stimulus of the rust was very marked. The development of spermogonia on leaves of primarily infected blackberries, to be noted later, is frequently inhibited, aecidia alone being formed. Nevertheless, by noting the areas where stomata occur in large numbers on the upper side one can tell several days in advance just where aecidia are to be developed. Whenever the rust at maturity covers only a part of the leaf it will be found that only that part is provided with additional stomata on the upper side. As long as the epidermis is in a plastic condition the advance of the mycelium into new areas is accompanied by the development of additional stomata.

The effect of the parasite on the production of stomata varies with the course of development of the rust. Under certain conditions, spermogonia may be distributed uniformly over the leaf from the first, but the formation of aecidia is long delayed or is entirely omitted. There will be about the same number of stomata on both sides of the leaf, the normal number occurring on the under side. It has also been found that when certain blackberries show the rust for the first time after primary infection by sowing sporidia of the short-cycled rust, the spermogonial stage is entirely lacking. The infected leaves show by their yellowish-green margins the extent to which the rust hyphae have penetrated. Aecidia will later develop along the discolored margins, then gradually spread toward the midrib, preceded by the fading out of the natural green and the production of stomata on the upper side. Very few spermogonia were found on some 50 plants of the varieties Kittatinny and Iceberg the first two years following infection; aecidia developed normally. Other varieties, Ancient Briton, Blowers, etc., developed spermogonia under similar conditions. The complete suppression of this stage in the life cycle of a rust is said to be of rare occurrence. A further study of the rust on different varieties of blackberry may serve to suggest a reason for the incomplete development or nonproduction of spermogonia by other rusts such as the one on the mallows.

In rare cases aecidia also fail to appear, most of the leaf merely becoming yellowish. Such leaves, though never bearing any spore forms, have been invaded by mycelium, as shown by the large number of stomata on the discolored areas of the upper side, the usual number appearing below.

The gametophytic mycelium of Gymnoconia does not always penetrate into every part of the leaf of, for example, the black raspberry. One-half only may be infected; variegations occur or angular patterns are laid out, the space between certain large veins escaping attack. Photographic prints can be made directly from these leaves, either before or after the chlorophyll has been extracted (Pl. 1). It will be found that production of stomata on the upper side of the leaf is coincident with the invasion of an area by the orange-rust hyphae. Plate 1, C, shows that the infected area of the raspberry leaf is sharply limited on the left side by the vein, and toward the tip on the right side the mycelium has spread out part way between the second and third veins. There were no stomata on the dorsal side of this leaf, except over the infected areas. A somewhat different pattern was worked out in the leaf shown as Plate 1, A. Dorsal stomata occurred on the left side except at the base, into which

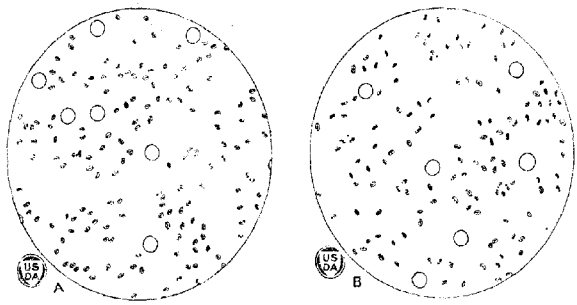


FIG. 1.—Relative numbers of stomata on corresponding areas from the ventral and dorsal sides of a leaf from an old cane of *Rubus occidentalis* infected with the long-cycled orange-rust. Position of stomata and spermogonia determined with the aid of a camera lucida; size and shape of stomata purely diagrammatic. In fact, the stomata on the dorsal side are on the average somewhat larger. Circles represent positions of spermogonia. The fields diagramed are such as were covered by a 16 mm. lens and Leitz Periplane 12x ocular. A, Area from ventral surface; B, Similar area from the dorsal side nearly opposite the area shown in A. There were 150 stomata in the area from the ventral side, and 136 in the dorsal area opposite. It would be necessary to examine several such fields before a single stoma would be found on the dorsal side of an uninfected leaf, or of an uninfected part of this same leaf.

hyphae had not penetrated. The lighter areas on the terminal and left lateral leaflets (Pl. 1, B), represent portions invaded and where dorsal stomata were numerous. The leaflets which escaped infection had no stomata on the upper side. Plate 1, F and G, shows prints of leaflets of Kittatinny blackberry on parts of which aecidia were just maturing. Practically the same number of stomata was found on the upper and lower sides on areas infected.

The time intervening between the opening of a particular infected leaf on new canes and the maturing of aecidia on that leaf becomes progressively shorter as the season advances, until a time is reached when fully formed aecidia are exposed as the leaf opens. What should be the effect of such an early appearance of aecidia on the underside of the leaf? The development of stomata on the underside is, just as we might expect, greatly interfered with, there being only a few normal stomata on that side, and these are at the tips of the serrations upon which no aecidia appear, just the reverse of the distribution of stomata in the normal leaf. An accurate count of the stomata has not been made on any considerable number of areas of normal and of infected leaves, but it is certainly inter-

esting to note that soon after the period is reached in a vigorous blackberry shoot when mature aecidia appear with the unfolding of the leaf, the tip of the new cane begins to outstrip the parasite in the upward growth, and the production of aecidia ceases; the grower says, "the plant is recovering."

That it is the gametophytic mycelium and not the sporophytic that has the power of inducing this morphological change in the host leaf is clear from a comparison of two sets of leaves, each bearing teleutosori. One collection was made July 10 at Lakewood, N. H. The leaves were thickly covered with spermogonia accompanied by an abundance of teleutosori, the aecidial stage having been suppressed. There were about the same number of stomata on both sides of the leaf. The other collection was made September 6 in Maine; these leaves, of course, bore only teleutospores. There were no stomata on the upper side, while the lower side was provided with what seemed to be the normal number. The occurrence of stomata on the upper side of leaves bearing spermogonia or aecidia could no doubt account for the infection of these leaves by aecidiospores. The two sorts of mycelium, systemic gametophytic and local sporophytic, not being antagonistic, develop their own reproductive bodies, spermogonia and teleutospores, side by side.

It has frequently come to the writer's attention that some of the earliest and most abundant development of teleutospores has occurred on leaves of old canes. These leaves must have been fully formed by the time aecidiospores were shed; this is especially true of systemically infected plants. Mountain blackberries harboring the orange-rust were transplanted from Maine to Maryland. In August, 1921, the leaves on the "new" canes bore an abundance of teleutosori, the "old" canes were defoliated and dying at this time. Early in June of the following year practically every leaf on the old canes bore teleutospores. These plants were watched again in 1923. Teleutospores first appeared on leaves of old canes which had previously borne spermogonia and frequently also aecidia. The position of the leaf on the cane, whether at the tip or at the base, was clearly of no great importance. The leaves on the new canes remained free from the telial stage during the summer of 1922. All leaves on the old canes had unfolded at about the same time and had borne aecidia, and would have been equally susceptible so far as maturity is concerned. On the other hand, leaves develop on the new canes one by one, as previously noted, so that as aecidiospores are shed leaves of different ages would be exposed. In this particular case in 1922 either the leaves on the new canes had not opened or they were not sufficiently mature when the spores were shed.

After seeing that leaves harboring the gametophytic mycelium were provided with additional stomata on the upper side, it occurred to the writer that this might account for the production of telia on so many leaves of the old infected canes. Aecidiospores were sowed on the upper side of leaves bearing spermogonia, the leaves then being placed in damp chambers. After two days the leaves were dropped in Flemming's fixture. The germ tube grows along the surface until it comes into the immediate vicinity of a stoma, then, if necessary, the end turns sharply, broadens out and sends the infection tube through the opening. The method of penetration was observed without sectioning by removing the chlorophyll from leaves killed in the Flemming's fluid. There were in the greenhouse on April 18 a number of potted plants of *Rubus occidentalis* systemically infected with the Gymnoconia, and now showing spermogonia. On many leaflets, especially on old canes, spermogonia

occurred only on a part of the leaf (Pl. 1, A). Aecidiospores were sowed over several plants in such a way that most of the spores fell on the dorsal side of leaves which had fully expanded. Many spores must have come in contact with the underside. The leaves on the tips of young basal shoots which had not unfolded were tagged. Particularly large numbers of spores were sowed on these folded leaves. The experiment was repeated April 25 with other plants. On May 25 teleutosori in abundance were found on plants from both sowings, showing that it takes at least four or five weeks in the greenhouse for the teleutosporic stage to reach maturity. Some of the leaves now bearing teleutosori also bore aecidia and spermogonia, while others bore only spermogonia; teleutospores were more abundant on such leaves.

In case aecidia or spermogonia occurred on only a part of a leaf, it was found that teleutosori were present only on that part, often on the upper as well as on the lower side. The part of the leaf not invaded by the gametophytic mycelium and free from the orange-rust stage had escaped infection when aecidiospores had been sowed on its dorsal surface. Leaves bearing teleutosori were decolorized and examined for stomata. The normal number appeared to be present on the ventral side of those leaves bearing only spermogonia and teleutosori. It was, of course, impossible to determine what had been the distribution of stomata on those areas on the ventral side of leaves where aecidia had destroyed the epidermis. Stomata were always found in abundance wherever teleutospores were present. Where aecidiospores of the Gymnoconia had been sprayed on both sides of leaves of old canes systemically infected, teleutosori first appeared on parts of leaflets having spermogonia. About 10 days later they began to appear among the hairs on the under side of neighboring leaves that had escaped invasion by the orange-rust hyphae. The results point clearly to the value of stomata on the upper side of leaves in facilitating the attack by the sporophytic germ tube. The gametophytic mycelium of the rust stimulates the host to provide ready means of access by the sporophytic stage which is to follow later.

As previously noted, the leaves of the black raspberry which are exposed to infection as the aecidiospores are being shed, may be grouped as follows:

1. Fully expanded but somewhat dwarfed leaves on old canes already infected with the systemic or orange-rust stage. The leaves or parts of leaves into which the gametophytic hyphae have penetrated will be rather devoid of hairs on the lower surface and have an abundance of stomata on the dorsal side, two factors favoring infection by the germ tubes.
2. Large, fully unfolded leaves on infected basal shoots from systemically infected plants such as produce the old canes in No. 1. Gametophytic hyphae evenly distributed throughout most of the leaflets, which will later usually be covered with aecidia. Very few hairs on the lower side, many stomata on the dorsal side.
3. Fully expanded leaves on normal old canes. Leaves somewhat tomentose on the underside. No stomata on the dorsal side (a very few at the tips of the serrations).
4. Very young leaves at the tips of systemically infected basal shoots. Some of these leaves will be tightly folded, others just expanding.
5. Fully expanded and growing leaves, tomentose beneath, on normal basal shoots.
6. Very young leaves, some still folded and very tomentose, others expanding, on basal shoots of normal canes.

The infection experiments described above show that some of the factors which determine the readiness with which a leaf of the raspberry can be infected by sowing aecidiospores resulting in the development of the teleutosori (*Puccinia peckiana*) are: Presence or absence of stomata

on the dorsal side, the maturity of the leaf, and the amount of tomentum covering the stomata. Leaves such as would come under groups 1 to 3 above are certainly most easily infected, and in the order given. Owing to the fact that it takes teleutosori a much longer time to mature on leaves of normal new canes, one is apt to be misled as to the relative susceptibility of the very youngest leaves. Very few teleutospores were ever developed on leaves which were still folded when the sowings of aecidiospores were made, even though these leaves were systemically infected, and therefore devoid of tomentum. The same line of infection experiments has also been carried on with blackberries and dewberries, with the same results. Teleutospores appear first and most abundantly if aecidiospores are sowed on leaves of the old canes that are systemically infected. The comparatively short time (four or five weeks) required for the development of teleutosori on leaves already harboring the orange-rust stage is accounted for by the rapidity with which such leaves reach maturity and fall off. The ease with which they may be infected is at least in part due to accessory dorsal stomata and freedom from tomentum.

Blodgett [3] reported that he had observed that blackberries bearing orange rust wilted sooner than uninfected plants, and that this was due to excessive transpiration.

Reed and Crabill [4] think that the greater loss of water by the rusted plant reported by Blodgett was due to the rupture of extensive areas of the ventral epidermis, which would facilitate evaporation. "Possibly other factors connected with the diseased condition may also operate to cause increased transpiration." These authors found that leaves of apples affected with Gymnosporangium transpire about the same whether they are in daylight or darkness. Healthy leaves transpire much more rapidly in daylight. The average for daylight and darkness, however, is practically the same for diseased and for healthy leaves. They think that the parasite must in some way affect the operation of the stomata. The substomatal cavities of rusted apple leaves are obliterated. Their figure 13 shows stomata only in the ventral epidermis of an area bearing spermogonia and aecidia.

In an isolated plant infected with Gymnoconia the chances for the production of telia are certainly vastly increased should the leaves which will some day have their ventral surface covered with blister-like aecidia be provided with stomata on the other side. Just this occurs as, we might say, a matter of safety, if not always of necessity, when leaves are systemically infected. Aecidia frequently fail to develop in leaves covered with spermogonia. In such a case there is no apparent reason why stomata on the ventral surface should not function properly. The accessory dorsal stomata are formed as the result of the stimulus of the gametophytic mycelium present just beneath the epidermis. The failure to produce aecidia following spermogonia is undoubtedly due to the condition of the host leaf, but it is not a condition under the control of the host. A great many investigations of the development and distribution of stomata have been made in the past, but the writer has been unable to find in the literature an account of another such curious interaction between host and parasite. There are several systemic rusts known which attack herbaceous plants. It would be interesting to know whether additional dorsal stomata are also developed by these hosts.

[3] BLODGETT, FREDERICK H. TRANSPIRATION OF RUST-INFESTED RUBUS. *In* Torreya, v. 1, p. 34-35. 1901.

[4] REED, HOWARD S., and CRABILL, C. H. THE CEDAR RUST OF APPLES CAUSED BY GYMNOSPORANGIUM JUNIPERI-VIRGINIANAE SCHW. Va. Agr. Exp. Sta. Tech. Bul. 9, 106 p., 23 fig. 1915. Bibliography, p. 104-106.

PLATE 1

The prints were made by bringing the leaves, from which the chlorophyll had been removed, in direct contact with Velox paper.

A.—Black raspberry leaflet, dorsal side. One-half of the leaflet, except a small portion of the lower left, infected and bearing spermogonia. Stomata on the upper side only where hyphae are present.

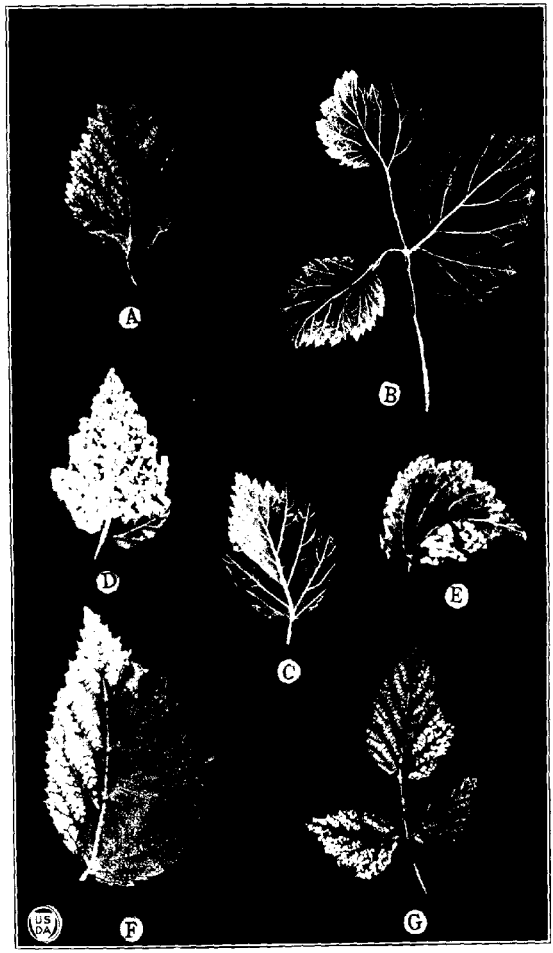
B.—Leaf of black raspberry, ventral surface. Left half of terminal leaflet infected; hyphae are beginning to invade the right half. Most of the left basal leaflet is infected; dark area along the midrib devoid of hyphae. No stomata were found on the dorsal side of the right basal leaflet, which was not infected, and none on the upper side of the others except where infected.

C.—Black raspberry, ventral surface. At the left the boundary line between infected and uninfected areas is very clear cut, being limited by a large lateral vein. On the right side the mycelium is advancing from the margin into the regions between the second and third veins; stomata present on the dorsal side only where leaf is infected.

D and E.—Leaflets from the same plant one month later; that is, one month after sowing aecidiospores of the Gymnoconia on the upper side of these leaves. Dorsal stomata and teleutosori only on areas now bearing aecidia. Teleutosori also on ventral side among aecidia.

F.—Leaf of Kittatinny blackberry accidia maturing. About the same number of stomata were found on both sides of the portions of the leaflets bearing aecidia. No stomata on dorsal side of basal leaflet at the right.

G.—Larger leaflet of Kittatinny blackberry, showing that the fungus advances from the margin toward the midrib. Dorsal stomata only on the area where aecidia are present on the ventral side. No spermogonia were formed.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